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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This proposal is designed to further our understanding of the role of bone marrow derived endothelial precursor cells to mammary tumor angiogenesis, tumor growth and progression. We have designed a unique animal model system to carefully study the involvement of these cells in mammary tumor progression and tumor angiogenesis. The potential importance of these cells to cancer warrants the analysis of these bone marrow derived vascular cells to as many types of cancer settings as possible. Moreover, studies have shown that these endothelial precursor cells are regulated by estrogen in endometrial angiogenesis, suggesting they may have an important role in the vascularization of mammary tumors and may be affected by anti-estrogen therapy.

Specific Aims:

1. To determine the relative contribution of bone marrow derived endothelial cells to naturally occurring mammary tumors during stages of early, late and metastatic tumor growth.
2. To determine the impact of the angiogenic cytokines VEGF-A and PlGF on tumor recruitment of bone marrow derived endothelial cells
3. To determine if estrogen impacts on the recruitment of bone marrow derived endothelial cells to mammary tumors

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Progress Report:

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INTRODUCTION:

This grant proposes to investigate a novel but important area of tumor angiogenesis that has particular relevance to estrogen regulated tissues like the breast. . Recent studies of bone marrow derived cells have strongly suggested that endothelial cell precursor cells can be mobilized from the bone marrow and incorporated into sites of ongoing robust angiogenesis in adults (1). Aside from the female reproductive system, such sites are usually sites of pathological angiogenesis such as that involved in tumor growth. Recent findings demonstrate that bone marrow derived endothelial precursor cells can be rate limiting for the growth of some tumors (2). In addition, these same cells contribute to uterine angiogenesis in an estrogen regulated manner (3).

This proposal is designed to further our understanding of the role of bone marrow derived cells to mammary tumor angiogenesis, tumor growth and progression. We have designed a unique animal model system to carefully study the involvement of these cells in mammary tumor progression and tumor angiogenesis. The potential importance of these

cells to cancer warrants the analysis of these bone marrow derived vascular cells to as many types of cancer settings as possible. Moreover, studies have shown that these endothelial precursor cells are regulated by estrogen in endometrial angiogenesis, suggesting they may have an important role in the vascularization of mammary tumors and may be affected by anti-estrogen therapy.

Task 1. To determine the relative contribution of bone marrow derived endothelial cells to naturally occurring mammary tumors during stages of early late and metastatic tumor growth (months 1-18)

a. Using bone marrow transplanted MMTV PyV-MT mice, quantitate the relative contributions of bone marrow derived endothelial cell precursor cells at different tumor states

b. Examine circulating bone marrow precursor cells with multiple markers during different stages of tumor progression using real-time RTPCR.

Our initial goal in Task 1b, to characterize circulating precursor cells using different markers has made the most progress thus far. We were fortunate to have a pathology resident joint this effort and characterize VEGFR-2, VE-Cadherin, AC133 and Tie-2 in the blood of human breast cancer patients and to correlate this to tumor stage. This work resulted in a publication which is in press, attached in the appendix in galley form (4). In short, we were surprised to find the marker that most clearly correlates to breast cancer progression was Tie-2. Because of this we are using Tie-2 more often as a marker for these cells and have obtained Tie-2 lac Z mice to use in our mouse studies.

Task 2. To determine the impact of the angiogenic cytokines VEGF-A and on tumor recruitment of bone marrow derived endothelial cells (Months 6-24)

a. Make and test regulation in vitro of stable transfected mammary tumor cells isolated from PyV-MT tumors containing tetracycline regulated expression vectors containing murine VEGF-A and PlGF.
b. Examine the relative contribution of bone marrow derived endothelial cells in orthotopic tumors made from the cell lines described above.
c. Determine if tumor secreted cytokines from the cell lines described above influence the release of endothelial cell precursors into the blood stream

We are currently isolating the cell lines with regulated VEGF and PlGF expression and will begin to obtain data from this task this summer.

Task 3. To determine if estrogen impacts on the recruitment of bone marrow derived endothelial cells to mammary tumors (Months 12-36)

a. Examine the impact of tamoxifen on bone marrow derived endothelial cells in naturally forming tumors of the MMTVPyV-MT mice

b. Examine the impact of tamoxifen on the circulating endothelial cell precursor cells
c. Make estrogen independent sublines from the VEGF-A expressing cell lines made in Task 2.
d. Determine if estrogen or tamoxifen impacts on the incorporation of bone marrow derived endothelial cells to these tumors in high versus low VEGF-A expressing tumors

KEY RESEARCH ACCOMPLISHMENTS:

- Optimization of protocol for real time RTPCR from human discarded blood
- Characterization of RNA expression for VEGFR-2, Tie-2, VE-Cadherin and AC133 in peripheral blood of patients with carcinoma in situ, infiltrating carcinoma or non-tumor controls.
- Writing and publishing paper reporting results
- Transfection of stable cell lines with VEGF and PlGF
- Purchase and breeding of Tie-2 lacZ mice for bone marrow transplant studies

REPORTABLE OUTCOMES:

Publication attached to appendix

CONCLUSIONS:

The research so far demonstrated that Tie-2 expression more than other markers of endothelial precursor cells correlates to breast cancer progression. This suggested that Tie-2 functions may be important for EPC function and stimulated us to turn our focus towards Tie-2 to a greater extent. To this end we have obtained a transgenic mouse with lac z expression driven by Tie-2 to use as a donor for bone marrow transplants. In addition to investigating VEGF and PlGF expression we are planning to clone and investigate the Tie-2 ligand also, Angiopoietin-1.

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Research Paper

Blood Markers for Vasculogenesis Increase with Tumor Progression in Patients with Breast Carcinoma

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KEY WORDS

Tie-2, Vasculogenesis, Breast cancer, EPCs

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ABSTRACT

Recent studies show that AC133—a hematopoietic stem cell antigen, when coexpressed with endothelial markers, identifies a population of endothelial precursor cells (EPCs) in peripheral blood that function in tumor vasculogenesis in animals. Little is known about whether EPCs contribute to human tumor vasculogenesis. We attempted to determine if, through increased peripheral expression of AC133 or endothelial markers previously associated with EPCs,VEGFR-2 and Tie-2, we could detect an EPC response in the blood of patients with breast carcinoma. Thirty patients were segregated based on their breast biopsy histology into infiltrating carcinoma, DCIS and control groups. Using Real Time PCR, we measured the expression of the aforementioned markers in reverse transcribed RNA extracts from preoperative peripheral blood specimens. The cancer patients had significantly elevated Tie-2 expression with the highest levels associated with infiltrating carcinoma. Our data suggest increased circulating EPC markers in tumor patients, but further study of this cell population is needed to better define its role in tumor vasculogenesis.

INTRODUCTION

In order for tumors to be able to grow and ultimately metastasize, they need to acquire the ability to form their own vasculature. This is accomplished by recapitulating the normal vessel-forming process already present in the host. Two mechanisms are responsible for vessel formation: angiogenesis —the growth of new vessels as sprouts from pre-existing vessels, and vasculogenesis—the de novo formation of vessels via the recruitment of endothelial precursor cells (EPC) which are present in the bone marrow and in the circulation.[1,2] Relatively little is known about vasculogenesis since, until recently, there has been no morphologic, immunologic, or molecular property that could reliably identify this cell population. However, Peichev et al.[3] demonstrated that AC133 a hematopoietic stem cell antigen, when co-expressed with a vascular specific marker such as VEGFR-2 or Tie-2, identifies these cells.

Several studies have demonstrated that EPCs play a role in tumor vasculogenesis in experimental animal models,[4-7] but there is currently no published data on whether or not this process takes place in humans. In this study, we attempted to determine if, by virtue of increased expression of genes for AC133, VEGFR-2, and Tie-2 in breast carcinoma patients, we could demonstrate an increase in circulating EPC in these patients which would suggest that a similar mechanism of vasculogenesis may play a role in the vascularization of human breast cancer.

MATERIALS AND METHODS

We collected EDTA-anticoagulated whole blood specimens from women prior to undergoing excisional breast biopsies or mastectomies. The clinical histories were reviewed, and patients with a documented history of chemotherapy or radiation therapy within the previous 12 months, surgery or trauma within the previous two weeks, or any non-breast malignancy at any time were excluded. Patients with previous breast malignancies were excluded if the histology on the current specimen was benign. Based on the resulting histology, the patients were segregated into three groups: infiltrating carcinoma (ductal and/or lobular), ductal carcinoma in situ (DCIS), and a control group with benign histology. For the control group, we also obtained specimens from demographically matched patients with no history of malignancy who were scheduled to undergo non-tumor surgery.

RNA was extracted from the specimens using the Qiagen protocol. The extract was reverse-transcribed, and the optical density at 260nm was measured to establish that a sufficient quantity of cDNA was available for PCR analysis. For the PCR, we designed our own primers for actin, AC133, VEGFR-2, Tie-2, and VE-cadherin using the Primer Express Software from ABI (Applied Biosystems, Foster City, CA). These were designed to cross introns and tested to ensure that they do not amplify DNA. The sequences were as follows:

TABLE 1 TUMOR HISTOLOGY AND AGE OF PATIENTS INCLUDED IN THE STUDY

	Number of Patients	Mean Age
Infiltrating Carcinoma		
Ductal: 9	12	64
Lobular: 2		
Ductal & Lobular features: 1		
DCIS	7	57
Control group		
Benign breast histology: 4		
* Florid ductal hyperplasia		
* Intraductal papilloma		
* Microcysts with calcifications	11	59
* Fibroadenoma		
Non-breast surgery: 7		
* Cosmetic procedures 2		
* Carotid endarterectomy: 2		
* Hiatal hernia repair		
* Tubal ligation		
* Lithotripsy		
Total patients	**30**	**61**

Actin: forward 5′-CCTGGCACCCAGCACAAT; reverse 5′-GGGCCGGACTCGTCATACT;

AC133: forward 5′-TGGATGCAGAACTTGACAACGT reverse 5′ATACCTGCTACGACAGTCGTGGT;

VEGFR-2: forward 5′-CACCACTCAAACGCTGACATGTA reverse5′-GCTCGTTGGCGCACTCTT;

Tie-2: forward 5′-GCTTGCTCCTTTCTGGAACTGT reverse 5′-CGCCACCCAGAGGCAAT;

VE-cadherin: forward 5′-TTTCCAGCAGCCTTTCTACCA reverse: 5′-GGAAGAACTGGCCCTTGTCA;

The detection method used was SYBR Green. Forty cycles of Real Time PCR using the ABI Prism 7700 were performed in triplicate on each sample. RNA levels were quantified using standard curves set up from each primer pair. The ratio gene expression was internally controlled by comparison to actin, and an unpaired two-tailed student t test was performed to establish statistical significance of the data.

RESULTS

A total of 30 patients were studied (Table 1). These included 12 infiltrating carcinoma patients (9 ductal carcinomas, 2 lobular carcinomas, and 1 with combined ductal and lobular features), 7 DCIS patients, and 11 controls (4 breast patients with benign histology and 7 non-breast patients). All patients were analyzed for the expression of the aforementioned markers (Fig. 1). The proteins AC133, VEGFR-2 and Tie-2 have been previously associated with EPCs, while VE-Cadherin is a marker of fully differentiated endothelial cells. Mild increases were observed in expression levels of AC133 and VEGFR-2 but VE-cadherin levels remained constant. A statistically significant increase was seen in the expression of Tie-2 in the infiltrating carcinoma relative to the control group.

DISCUSSION

Our findings demonstrate a clear increase in peripheral Tie-2 expression in breast carcinoma patients with a smaller increase in AC133 and VEGFR-2 expression in these patients. Possible reasons for this difference may include variations in EPC marker expression at the stage of development in which they were studied, namely in the peripheral circulation following release from the bone marrow but prior to homing in on the tumor site. Recent studies have shown that the Tie-2 ligand potently stimulates EPC release in animal models.[8] Our data suggest that Tie-2 levels may be the easiest to use as markers of vasculogenesis in breast cancer. While these data suggest a role for EPC in the process of tumor vasculogenesis, additional studies which incorporate other modalities such as flow cytometry and immunohistochemistry, as well as other involved tissues such as bone marrow and tumor tissue, are needed to better define the properties and functions of this cell population. Additionally, Tie-2 expression in the blood of cancer patients may be a much-needed and tractable surrogate marker for anti-angiogenesis therapy.

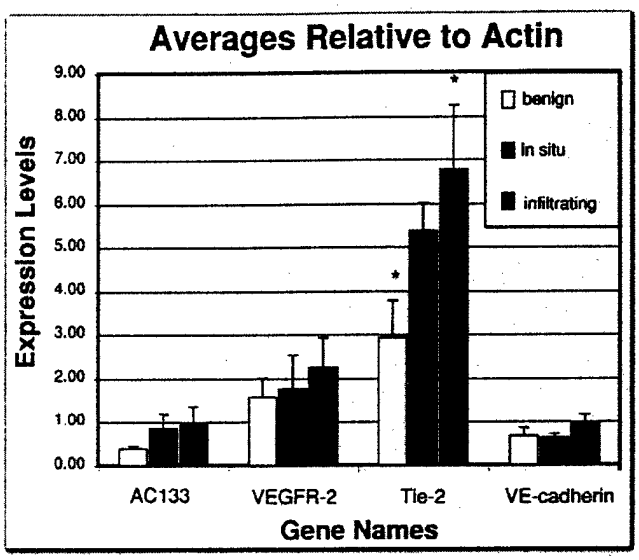

Figure 1: Relative expression levels of EPC markers increase in the blood of breast cancer patients. Quantitative RNA levels by real-time RT-PCR are presented relative to Actin and grouped by tumor histology. *p = 0.038.

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